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(54) Title: SCANNING PROBE MICROSCOPY IMMUNOASSAY

(57) Abstract

Methods and test kits for detecting the presence of an analyte in a test sample on a molecule-by-molecule basis by using scanning probe microscopy, in which the test sample suspected of containing the analyte of interest is exposed to a test piece to which an analyte specific substance has been attached, and the test piece is scanned by scanning probe microscopy to determine the presence or absence of the analyte.

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SCANNING PROBE MICROSCOPY IMMUNOASSAY

Background of the Invention

This invention relates generally to the detection of analytes, and more particularly, relates to the detection of analytes by utilizing scanning probe microscopy.

The two most widely known and used scanning probe microscopy (SPM) techniques involve the use of either the Scanning Tunnelling Microscope (STM) or the Atomic Force Microscope (AFM), also known as the Scanning Force Microscope (SFM). These techniques have been the subject of many scientific publications and review articles. See, for example, P. K. Hansma et al.,"Scanning Tunneling Microscopy and Atomic Force Microscopy: Application to Biology and Technology" Science 242: 209 - 216 (1988); L. Feng et al., "Scanning Tunneling Microscopy of Proteins on Graphite Surfaces" Scanning Microscopy 3:399-410 (1989); J. D. Baldeschwieler et al., "The Scanning Probe Microscope: A Powerful Tool For Visualizing the Micro World" American Laboratory:34-39 (February, 1991), as well as articles cited in these three publications. STM and AFM instruments are commercially available from several sources, such as Digital Instruments, Inc. (Santa Barbara, CA 93117), TopoMetrix (Pasadena, CA 91106), and EG&G Princeton Applied Research (Princeton, NJ 08543).

In Scanning Tunnelling Microscopy (STM), the oldest known SPM method, the probe is electrical. At each X, Y coordinate position, the sensor, a very fine wire drawn to an atomically sharp tip, is maintained a constant distance above the surface by a piezoelectric ceramic driver. Typically, a constant potential is maintained between the tip and the surface to be examined; hence, the requirement that the substrate be conductive. Conductive substrates such as Highly Oriented Pyrolytic Graphite (HOPG) (available from Digital Instruments, Santa Barbara CA) and gold have been used. Crystals also can be examined, since they are usually conductive. At each X, Y position, the Z coordinate at a constant distance above the surface is recorded using the tunneling current from the tip to the surface in a feedback circuit to keep the tunneling current constant, and hence, the distance from the tip to

surface constant. The "map" resulting from such measurements consists of the height, Z, of the surface at various X, Y coordinates. For conductive substrates, this method allows the resolution of atoms in crystalline latices (Hansma et al., 1988, supra).

Originally, it was postulated that only conductive molecules could be imaged via STM. Thus, for example, M. Amrein et al, Science, 240: 514 - 516 (1988) used STM to image a complex between DNA and recA, a protein involved in recombination. However, the sample had been coated with a conductive platinum, iridium and carbon film. More recently, T. P. Beebe, Jr., et al., Science, 243: 370 - 372 (1989) imaged uncoated double-stranded DNA deposited on a graphite surface with an STM. Moreover, STM has been used to image uncoated Z-DNA on graphite, P. G. Arscott, et al, Nature, 339: 484 - 486 (1989).

STM can be used to image samples immersed in an aqueous solution. S. M. Lindsay et al, Science, 244: 1063 - 1064 (1989) electrophoretically deposited double-stranded DNA onto a gold surface and observed the double helix conformation of the macromolecule in an aqueous environment. The use of STM in ultra high vacuum has allowed the imaging of DNA at the atomic level with DNA dried onto HOPG, as reported by R. J. Driscoll et al, Nature, 346: 294 - 296 (1990).

Biological molecules other than DNA have been imaged by STM. L. Feng et al., J. of Colloid and Interface Science 126:650-653 (1988) have imaged Human Serum Albumin adsorbed onto HOPG, and were able to observe the three domains of the molecule. Also, R. D. Edstrom et al, Biochemistry: 28: 4939 - 4942 (1989) imaged Phosphorylase Kinase and Phosphorylase b, two enzymes which are involved in energy utilization in muscle. The proteins were dried from concentrated solutions (1 mg/mL) onto graphite before scanning the dry samples at atmospheric pressure. Both Feng et al. and Edstrom et al. were able to observe known features of the proteins they were imaging.

Recently, however, a caution to the interpretation of images of materials deposited on HOPG has been published. C.R. Clemmer and T.P. Beebe, Jr.noted that there frequently are structures on HOPG which may be misinterpreted as DNA or proteins adsorbed on the surface. C.R.

Clemmer and T.P. Beebe, Jr., "Graphite: A Mimic for DNA and Other Biomolecules in Scanning Tunneling Microscope Studies" Science 251:640-642 (1991)

Atomic Force Microscopy (AFM) is usually performed by observing the deflection of a microscopic spring as a probe is lowered to the surface of the material to be examined. The spring ends in a fine tip and its deflection by contact with the surface can be observed by monitoring a beam of laser light reflected from a mirrored surface on the back of the probe. AFM does not require a conductive substrate, and further it does not require that the material to be imaged be conductive. The former allows non-conductive materials such as plastic, glass, and silicon which are compatible with various biochemical and biomedical processes to be used. The latter allows the direct detection of various kinds of biological molecules without the necessity of coating them with a conductive layer. Unfortunately, the contact nature of AFM may perturb the sample itself, thus allowing only a distorted image of the material on the surface. For example, J. N. Lin et al., Langmuir 6: 509-511 (1990) adsorbed a mouse monoclonal antibody onto mica and monitored the reaction with AFM. The dominant features observed were "aggregates" of antibody at the initiation of the reaction which changed into "ridges" as the monitored reaction proceeded. The authors reported that it was clear that the tip of the probe had been "massaging" or pushing the molecules on the surface. AFM also has been useful for studying conductive molecules at high resolution. S. Manne et al., Science, 251: 183 - 186 (1991) have reported on the imaging at atomic resolution of gold electrodes with copper deposited on them.

Probes other than electron tunneling or atomic force can be used to explore the surfaces of various substrates. For example, atomic scale friction can be measured in a Scanning Force Microscope, as well as surface "hardness" or "softness". Electrostatic interactions also provide another probe of a surface; the sensor is essentially an AFM probe, but with a charge (positive or negative) imparted to its surface. Electrostatic repulsion or attraction is measured by deflection of the probe as it approaches the surface giving a charge map of the surface.

Although the need for specifically and reproducibly presenting samples to the scanning probe has been stated (for example, by P. K. Hansma et al., supra), there previously has been no suggestion or attempt at utilizing specific ligand-ligand interactions (such as antibody-antigen or nucleic acid hybridization) to accomplish this end. K. Eric Drexler and John S. Foster, Nature 343: 600 (1990) have mentioned the use of ligand-ligand interactions in the context of SPM, but only as a speculative proposal to effect a specific chemical reaction on a molecule at a selected site. Heretofore, there has there been no suggestion that SPM methods such as STM or AFM can be useful in measuring the product of a specific binding reaction (such as antibody-antigen or nucleic acid hybridization). This invention provides novel methods which can be utilized to accomplish these results, heretofore unknown. Also provided in the present invention are means to firmly immobilize samples to the substrate, thereby obviating the problems observed by Lin et al., supra, in which the probe moved the sample making detailed structural determination impossible.

Summary of the Invention

The present invention provides a first method for determining the presence and/or amount of an analyte in a test sample, which method comprises contacting a test sample with a test piece to which an analyte specific substance has been attached for a time and under conditions sufficient to allow binding of the analyte to the test piece, and scanning the test piece by scanning probe microscopy to determine the presence or absence of the analyte. Quantitation of the amount of analyte present in the test sample can be accomplished by counting the number of analyte molecules in one or more fields of the test piece and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically. Scanning is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane such as to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates. Scanning may be performed either by a scanning tunnelling microscope or an atomic force microscope The analyte specific substance is attached to the test piece by adsorption or by covalent binding. The preferred solid phases of the test piece used for

adsorption attachment are plastic or metal. The preferred solid phases of the test piece used for covalent attachment are derivatized plastic, metal, glass and silicon. The analyte specific substance can be an antibody, an antigen, an antigen analog, DNA, RNA, a lectin, a hapten, avidin, biotin, folate binding protein, or intrinsic factor.

The present invention also provides a second method of determining the presence of an analyte which may be present in a test sample, which method comprises (a) mixing the test sample with a known amount of its specific binding partner to form a mixture; (b) applying the mixture to the test piece to which the analyte or an analyte analog has been attached, for a time and under conditions sufficient for a reaction to occur; and (c) scanning the test piece by scanning probe microscopy to determine the presence and/or amount of the specific binding partner, wherein the presence and amount of the specific binding partner bound is inversely proportional to the amount of analyte present in the test sample. Quantitation of the amount of analyte present in the test sample can be accomplished by counting the number of specific binding partner molecules in one or more fields of the test piece and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically. Scanning is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane such as to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates. The analyte specific substance is attached to the test piece by adsorption or by covalent binding. The preferred solid phases of the test piece used for adsorption are plastic or metal. The preferred solid phases of the test piece used for covalent attachment are derivatized plastic, metal, glass and silicon. The analyte specific substance can be an antibody, an antigen, an antigen analog, a hapten, DNA, RNA, a lectin, avidin, biotin, folate binding protein or intrinsic factor.

Also provided are test kits useful for determining the presence of an analyte suspected of being present in a test sample by scanning probe microscopy, comprising a test piece having bound thereto an analyte specific substance, an analyte, or an analyte analog. The analyte specific

substance, analyte or analyte analog preferably is covalently attached to the test piece of the test kit. The test piece of the test kit preferably comprises a solid phase,the solid phase being selected from the group consisting of derivatized plastic, glass, silicon and metal. The test piece of the test kit most preferably comprises a silicon wafer.

Brief Description of the Drawings

FIG 1A is an SPM image of an amine-activated silicon test piece.

FIG. 1B is a graph of the profile of the surface of an amine-activated silicon test piece wherein height (in angstroms [Å]) of the surface of the test piece is plotted against surface length (in nanometers [nm]).

FIG. 2A is an SPM image of an activated silicon test piece to which an analyte specific substance (anti-Hepatitis B surface antigen monoclonal antibody) has been attached.

FIG. 2B is a graph of the profile of the surface of an amine-activated silicon test piece to which an analyte specific substance (anti-Hepatitis B surface antigen monoclonal antibody) has been attached, wherein height (in angstroms [Å]) of the surface of the test piece is plotted against surface length (in nanometers [nm]).

FIG. 3A is an SPM image of an activated silicon test pieces to which an analyte specific substance (anti-Hepatitis B surface antigen monoclonal antibody) has been attached, after reaction with an analyte (r Hepatitis B surface antigen).

FIG. 3B is a graph of the profile of the surface of an amine-activated silicon test piece to which an analyte specific substance (anti-Hepatitis B surface antigen monoclonal antibody) has been attached, after reaction with an analyte (r Hepatitis B surface antigen), wherein height (in nanometers [nm]) of the surface of the test piece is plotted against surface length (in nanometers [nm]).

FIG. 4 is an SPM image of an amine-activated silicon test piece to which an analyte specific substance (anti-Hepatitis B surface antigen monoclonal antibody) has been attached, after reaction with an analyte (r Hepatitis B surface antigen).

Detailed Description of the Invention

The present invention provides a means for specifically binding various analytes to a surface and a molecule by molecule

determination of the presence or absence of the analyte in question using Scanning Probe Microscopy techniques. The present invention thus provides the application of a powerful new analytical tool to the problem of determining the presence or absence of specific molecules. The molecules are typically, though not limited to, molecules having biological properties such as antibodies, antigens, drugs, and the like. The molecules can be nucleic acids such as DNA and RNA. Or, supramolecular structures consisting of assemblages of macromolecules such as virus coat protein particles, viruses, bacteria, membranes or membrane fragments, cells or fragments of cells can be the subject of the current invention. The determination of the presence of the molecule of interest also can result in the quantitation of the amount of analyte present, if desired.

The analytical tool used in this invention is termed Scanning Probe Microscopy (SPM) and consists of a related family of techniques which can determine the properties of a surface at the molecular and even atomic level. In this assay technique, a micro-sensor is rapidly and systematically scanned across a surface in (for example) the X direction. Upon the completion of one pass, the sensor is advanced a small distance in the Y direction, and another trace is made back in the X direction, thereby defining an X, Y plane. The response of the sensor is monitored as it is rastered (i.e., systematically scanned) across the surface. Customarily, the sensor response is utilized in a feedback loop to control the Z position of the sensor. In this fashion, a complete map is made of a function of the surface at various X, Y coordinates. If an STM is used, the microsensor detects current flowing across the gap between the surface and the microsensor. The current flow is used in a feedback loop to control the height of the senor above the surface so as to keep the current flow at a preselected value. West et al., U. S. Patent No. 4,952,857, which is incorporated herein by reference, report an improved STM which utilizes an adaptive feedback control circuit. If an AFM is used, the microsensor physically contacts the surface, and the displacement of the sensor is used in a feedback loop to control the height of the sensor above the surface.

The present invention provides an immunoassay which utilizes specific binding members. A "specific binding member," as used herein,

is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA molecules. The term "hapten", as used herein, refers to a partial antigen or non-protein binding member which is capable of binding to an antibody, but which is not capable of eliciting antibody formation unless coupled to a carrier protein.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as, an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. As a member of a specific binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of intrinsic factor protein as a member of a specific binding pair for the determination of Vitamin B12, the use of folate-binding protein to determine folic acid, or the use of a lectin as a member of a specific binding pair for the determination of a carbohydrate. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances. The details for the preparation of such antibodies and the suitability for use as specific binding members are well known to those skilled in the art.

The types of molecules and supra-molecular structures to be assayed by this invention are those customarily measured by ligand-ligand interactions. Macromolecular antigens most often have been measured in "sandwich" immunoassay measurements which utilize one antibody to "capture" the analyte and another antibody having a measurable signal generating compound (measurable detectable label) to allow detection of the reaction by determining the signal produced. Signal generating compounds (labels) have included radioisotopes, fluorophores, chemiluminescent compounds and enzymes, whose presence can be determined by the conversion of appropriate substrates to chromogens, fluorophores, or light.

Low molecular weight antigens conventionally have been detected by competitive binding immunoassays such as Radioimmunoassay (RIA), Fluorescence Polarization Immunoassay (FPIA), and Enzyme Multiplied Immunoassay Technique (EMIT). Nucleic acids have been detected by various hybridization techniques frequently following an amplification procedure such as the Polymerase Chain Reaction (PCR) or the Ligase Chain Reaction (LCR).

Supra-molecular structures frequently have been determined by binding specific binding agents such as antibodies with a measurable signal generating compound (measurable detectable label) to surface markers and observing the measurable bound label after removal of excess reagent. Alternatively, features internal to the structure could be stained allowing observation of the particle. Specificity in the latter case can be imparted by immunologically trapping the structure using specific binding agents such as antibodies against surface markers. Thus, supramolecular analytes such as cells, malignant cells, blood cells, sperm, and microorganisms including bacteria, viruses, parasites, rickettsia, fungi, mycobacterium, mycoplasma, may be analyzed by the method of this invention.

Test samples which may be tested by the methods of the invention includes both liquid and solid substances of biological and non-biological origin. Liquid and/or solid samples include those of biological origin, for example, human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, biological fluids such as cell culture

supernatants, tissue specimens and cell specimens, and the like. Tissue specimens and cell specimens do not need to be fixed in order to be assayed by the method of the invention. Also contemplated are liquid and solid non-biological specimens including soil samples, water samples, oil samples, and the like.

A test piece comprising a solid phase may be used according to the method of the invention. A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

It is contemplated and within the scope of the invention that the test piece comprising a solid phase also can comprise any suitable porous material. By "porous" is meant that the material is one through which the test sample can easily pass. In the present invention, the solid phase can include Nucleopore™ membranes (available from VWR Scientific, Chicago, IL) and other porous surfaces such as woven nylon polyethylene and polypropylene mesh membranes made by Spectrum Medical Industries, Inc., Los Angeles, CA) or other porous or open pore materials well known to those skilled in the art (e.g., polyethylene sheet material). The solid phase can also comprise polymeric or glass beads, microparticles, tubes, sheets, plates, slides, wells, tapes, test tubes, or the like.

Although the details provided herein are focused on an immunological procedure, the extension to other specific ligand-ligand interactions is contemplated and within the scope of the invention. Also contemplated and within the scope of the invention is the use of surface probes other than Electron Tunnelling or Atomic Force, such as atomic friction, scanning magnetic, scanning thermal or other scanning micromechanical instruments, when used in a scanning mode.

The use of SPM to monitor specific binding reactions can occur in many ways. In one embodiment of the invention, one member of a specific binding partner (analyte specific substance) is attached to a surface suitable for scanning. The attachment of the analyte specific substance may be by adsorption to a test piece which comprises a solid phase of a plastic or metal surface, following methods known to those of ordinary skill in the art. Or, covalent attachment of a specific binding partner (analyte specific substance) to a test piece which test piece comprises a solid phase of derivatized plastic, metal, silicon, or glass may be utilized. Covalent attachment methods are known to those skilled in the art and include a variety of means to irreversibly link specific binding partners to the test piece. If the test piece is silicon or glass, the surface must be activated prior to attaching the specific binding partner. Activated silane compounds such as triethoxy amino propyl silane (available from Sigma Chemical Co., St. Louis, MO), triethoxy vinyl silane (Aldrich Chemical Co., Milwaukee, WI), and (3-mercapto-propyl)-trimethoxy silane (Sigma Chemical Co., St. Louis, MO) can be used to introduce reactive groups such as amino-, vinyl, and thiol, respectively. Such activated surfaces can be used to link the binding partner directly (in the cases of amino or thiol) or the activated surface can be further reacted with linkers such as glutaraldehyde, bis (succinimidyl) suberate, SPPD 9 succinimidyl 3-[2-pyridyldithio] propionate), SMCC (succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate), SIAB (succinimidyl [4-iodoacetyl] aminobenzoate), and SMPB (succinimidyl 4-[1-maleimidophenyl] butyrate) to separate the binding partner from the surface. The vinyl group can be oxidized to provide a means for covalent attachment. It also can be used as an anchor for the polymerization of various polymers such as poly acrylic acid, which can provide multiple attachment points for specific binding partners. The amino surface can be reacted with oxidized dextrans of various molecular weights to provide

hydrophilic linkers of different size and capacity. Examples of oxidizable dextrans include Dextran T-40 (molecular weight 40,000 daltons), Dextran T-110 (molecular weight 110,000 daltons), Dextran T-500 (molecular weight 500,000 daltons), Dextran T-2M (molecular weight 2,000,000 daltons) (all of which are available from Pharmacia, LOCATION), or Ficoll (molecular weight 70,000 daltons (available from Sigma Chemical Co., St. Louis, MO). Also, polyelectrolyte interactions may be used to immobilize a specific binding partner on a surface of a test piece by using techniques and chemistries described by pending U. S. Patent applications Serial No. 150,278, filed January 29, 1988, and Serial No. 375,029, filed July 7, 1989, each of which enjoys common ownership and each of which is incorporated herein by reference. The preferred method of attachment is by covalent means.

Following attachment of a specific binding member, the surface may be further treated with materials such as serum, proteins, or other blocking agents to minimize non-specific binding. The surface also may be scanned either at the site of manufacture or point of use to verify its suitability for assay purposes. The scanning process is not anticipated to alter the specific binding properties of the test piece.

It is preferred that the analyte to be tested be bound to a test piece. Test pieces contemplated include surfaces of glass, plastic, metal and silicon. Most preferably, the test piece comprises a silicon wafer. The wafer can be of varying thickness. Therefore, silicon wafers from about 10 to about 30 mils can be used as the test piece. Preferably, the silicon wafer will be of a thickness of about 18 to about 22 mils. The silicon wafer is sliced from a large crystal and can have an orientation of (100) or (111). Most preferably, a silicon wafer such as described will be a polished, monocrystalline silicon slice.

To perform an assay, the test piece to which an analyte specific substance has been attached is contacted with a test sample for an adequate period of time under conditions sufficient to allow the binding of the analyte specific binding partner, if present in the test sample, to the analyte specific substance. Upon completion of the reaction, the test piece is scanned by scanning probe microscopy methods described herein to determine the presence or absence of the analyte. A micro-sensor is

rapidly and systematically scanned across a surface in (for example) the X direction. Upon the completion of one pass, the sensor is advanced in a small direction in the Y direction, and another trace is made back in the X direction. The response of the sensor is monitored as it is rastered (i.e., systematically scanned) across the surface. Customarily, the sensor response is utilized in a feedback loop to control the Z position of the sensor. In this fashion, a complete map is made of a function of the surface at various X, Y coordinates.

If quantitation of the analyte is desired, the number of molecules in a particular field is counted and, either by reference to previously measured standards or mathematically, the concentration in the sample is determined. Multiple fields on the same test piece may be examined to extend the sensitivity and accuracy of the method. Before the sample is scanned, the test piece may be washed and dried or washed and scanned wet. Optionally, the test piece may be scanned with the sample still in place. The probe will sense only those molecules tightly bound at the surface of the test piece; therefore, weakly bound molecules will not interfere. It can be anticipated that the probe itself will sweep the surface clean of lightly bound interfering molecules.

Alternately, at the convenience of the practitioner of the invention, the sample containing the analyte may be mixed with a known amount of its specific binding partner and the mixture applied to the test piece to which the analyte or an analyte analog has been attached. This amount of specific binding partner can be determined prior to beginning the assay. This so-formed mixture is incubated for a time and under conditions sufficient for a binding reaction to occur. Again, upon completion of the reaction, the test piece is scanned as previously described for the presence and amount of the specific binding partner. The presence and amount of the specific binding partner bound will be inversely proportional to the amount of analyte in the sample. As previously stated, the sensitivity and accuracy of the measurement process may be enhanced by scanning multiple fields of the same test piece.

If the analyte is a polynucleic acid such as DNA or RNA, the appropriate specific binding partner is a polynucleic acid complementary

to one or more target sequences suspected of being present in the test sample. Such methods are well known in the art. See, for example, Falkow et al., U.S. Patent No. 4, 358,535 and Ranki et al., U.S. Patent No. 4, 486,539, both of which are incorporated herein by reference Attachment of the capture sequence onto the test piece may be achieved covalently as, for example, described by Stabinsky, U.S. Patent No. 4,751,177, which is incorporated herein by reference Alternatively, the capture sequence may be derivatized with a hapten, which in turn may be bound to the surface of the test piece through an antibody or the like as described by Snitman et al., published as International Patent Publication WO 86/07387, which is incorporated herein by reference. The hapten derivatized capture sequence may be mixed with the sample prior to exposure to the test piece or it may be pre-bound to the antibody activated test piece. The antibody may be attached as described above. Prior to its exposure to the test piece, the target sequence may be amplified by use of the polymerase chain reaction (PCR) or ligase chain reaction (LCR) such as to lessen the number of fields which need to be scanned to determine the presence or absence of analyte.

U. S. Patents No. 4,683,195 and 4,683,202 teach a method of amplifying DNA sequences by using PCR. Both of these patents are incorporated herein by reference. Briefly, in PCR, two complementary polynucleotide strands are amplified by treating the strands with two oligonucleotide primers such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand. The primers are selected such that the extension product of one primer forms a template for the synthesis of an extension product from the other primer once the extension product of the one primer is separated from the template. A chain reaction is maintained by a cycle of denaturing the primer extension products from their templates, treating the single-stranded molecule generated with the same primers to re-anneal, and allowing the primers to form further extension products. The cycle is repeated for any many times as it takes to increase the target nucleic acid segments to a concentration where they can be detected. The amplified target sequence can be detected by denaturing the double-stranded products formed by PCR, and treating those products with one or more reporter probes which hybridize with the extension products.

The Ligase Chain Reaction (LCR) amplifies sections of polynucleic acid by copying the section of polynucleic acid, and copying the copies of that section of polynucleic acid, many times over. This method is described in European Patent Application No. 0 320 308 published June 14, 1989, which is incorporated herein by reference. In this procedure, two probes (for example, A and B) complementary to immediately adjacent regions of a target sequence are hybridized and ligated. This ligated probe then is denatured away from the target, after which it is hybridized with two additional probes (A' and B') of sense opposite to the initial probes A and B. The secondary probes are themselves then ligated. Subsequent cycles of denaturation/hybridization/ligation create the formation of double-length probes of both sense (+) and antisense (-).

In LCR, the nucleic acid of the sample is provided either as single stranded or as double-stranded polynucleic acid which is denatured to separate the strands. Four probes are utilized: the first two probes (A and B) are the so-called primary probes, and the second two probes (A' and B') are the so-called secondary probes. The first probe (A) is a single strand capable of hybridizing to a first segment of the primary strand of the target nucleotide sequence. The second probe (b) is capable of hybridizing to a second segment of the primary strand of the target nucleotide sequence. The 5' end of the first segment of the primary strand of the target is positioned relative to the 3' end of the second segment of the primary strand of the target to enable joining of the 3' end of the first probe to the 5' end of the second probe, when the probes are hybridized to the primary strand of the target nucleotide sequence. The third probe (A') is capable of hybridizing to the first probe, and the fourth probe (B') is capable of hybridizing to the second probe (B). The hybridized probes are ligated to form reorganized fused probe sequences. Then, the polynucleic acid in the sample is denatured to separate ligated probes from sample polynucleic acid. Successive cycles wherein the ligated probes and target polynucleic acid undergo the above-described process are performed to increase the amount of detectable polynucleic acid in the sample. The amount of cycles performed is dependent upon the sequence used and the sensitivity required of the test. Usually, the cycle can be repeated from 15 to 60 times.

The sensitivity of SPM immunoassays may be enhanced by use of standard immunological methods appropriately adapted to the type of probe used. For example, if the antigen captured by specific antibody binding has an epitope available for binding a second antibody, a second antibody labelled with materials such as colloidal metals, colloidal non-metals, small latex particles, charged polymers, liposomes, fixed cells, and the like can be used for enhanced detection of the analyte. For competitive assays when the analyte or an analog is attached to the surface, the specific binding ligand used in limited amounts may be appropriately labelled. For polynucleic acid analytes, in addition to the complementary capture sequence, a complementary, non-overlapping, label sequence can be used. In addition to the labels mentioned above, long sequences of double stranded DNA, linear or circular, may be used to enhance the sensitivity of the method.

By proper selection of the specific binding partner, a high degree of specificity can be imparted to a Scanning Probe Microscope Assay. That is, if an antibody, monoclonal or polyclonal, with very low cross-reactivity, is used to bind analyte to the surface of the test piece, it is anticipated that by proper combination of washings and probe manipulations a high degree of specificity will be exhibited by the assay. That is, only the desired analyte will be specifically immobilized on the surface of the test piece and so the detection of any material bound incremental to the specific binding partner originally attached to the surface will constitute a positive binding event. Additional specificity can be built into the assay if an additional requirement for a binding event to be scored as a positive result is used. Since the scanning probe microscope affords information on the size, shape and other characteristics can be used to provide additional specificity requirements for the assay result to be regarded as positive. For example, in an assay for Carcino-Embryonic Antigen (CEA), if material from the sample which is bound to the test piece does not conform to the established parameters of the CEA molecule (a multi-domain, heavily glycosylated molecule of approximately 180,000 daltons), then the test result will be scored as negative, thus avoiding a "false positive" result. Methods and algorithms for automatically analyzing images with regard to shape and size are well known in the field of image analysis. Neural network methods may be advantageous for the identification of analytes on the basis of their size and shape in

comparison to known samples. Scanning Probe Microscopy Immunoassay (SPMIA) offers both necessary (binding to surface) and sufficient (size, shape, other characteristics) conditions to make the assays very resistant to erroneous results.

In the case of polynucleic acid assays, i.e., for DNA or RNA, stringency conditions will be used which limit the non-specific binding of target to capture probe. Commonly used stringency conditions include high temperature, denaturing surfactants, organic solvents, high ionic strength buffers and the like. If a capture probe is used, it must be of sufficient length to allow efficient binding under conditions of high stringency. It is anticipated that there will be limited information available from the size and shape of the captured target sequence relevant to establishing sufficiency conditions to allow additional specificity in the assay. However, as pointed out by Driscoll et al. (supra), SPM allows atomic resolution of double stranded nucleic acids, thereby allowing verification of the "fit" of the target to capture sequence as well as confirmation that the sequence is that which is expected .

Further, it is contemplated that means of accelerating the binding reaction will provide shorter assay time. Temperature changes wherein the temperature used to affix the analyte specific substance to the test piece, as well as, the use of polyethylene glycol, ultrasonic exposure and other means, each may contribute either alone or in combination to the acceleration of the reaction. Reactions of macromolecular and supramolecular analytes can be accelerated by conducting the reaction in an ultracentrifuge wherein the analyte is sedimented onto the surface of the test piece at high gravitational force. Non-bound material can be removed by washing, and the specifically bound material analyzed by SPM. These accelerating means are contemplated to be within the scope of the present invention.

If quantitation of the analyte is desired, the number of molecules in a particular field is counted and, either by reference to previously measured standards or mathematically, the concentration in the sample is determined. Thus, if a reaction is at equilibrium with high affinity binders on the surface, it is possible to calculate the number of antigens which on average will be in each field. For example, if a 50 μL sample of

Hepatitis B Surface Antigen at 1 ng/mL is applied to a 8 mm x 8 mm test piece, there will be approximately 15 x 10 6 particles (MW= 2 x 106 daltons) per test piece. On average, there will be 0.23 particles per 1 μ x 1 μ field. By counting multiple fields and applying known statistical methods such as the binomial distribution, it is possible to accurately determine the concentration of analyte. It is contemplated that algorithms can provide a means for counting objects in the field, thereby automating quantitation of the number of objects in the field.

The present invention will now be described by way of Examples, which are meant to illustrate, but not to limit, the spirit and scope of the invention.

EXAMPLES

Example 1

Preparation of Amine Activated Test Pieces

Polished monocrystalline silicon slices 0.5 mm thick (100) orientation and n type (obtained from Unisil Corporation) were diced into 8 mm squares. The test pieces were immersed in a 10% (v/v) solution of triethoxy amino propyl silane (Sigma Chemical Co., St. Louis, MO) in water for 15 minutes at room temperature. The pieces then were washed with a stream of distilled water from a laboratory squeeze bottle for approximately five (5) seconds, and blown dry with a nitrogen stream. The drying was accomplished by gripping a test piece with tweezers at one corner and then directing a gentle stream of nitrogen across the surface of the test piece aimed toward the tweezers. Drying was continued until all visible drops of water were removed from the surface which then exhibited a shiny, highly reflective surface with no visible residue.

Example 2

Coating of Test Pieces With Antibody

A solution of murine monoclonal antibody directed against Hepatitis B Surface Antigen (anti-HBsAg) (IgG1 subtype) (available from Abbott Laboratories, Abbott Park, IL in the commercially available kit AUZYME® MONO) was prepared in 2-[N-morpholino] ethane sulfonic acid (MES) (Sigma Chemical Co., St. Louis, MO) buffer (50mM, pH 7.0) at a concentration of 400 μg/mL. A solution of 1-ethyl-3-(3-

dimethylaminopropryl) carbodiimide (EDAC) (Aldrich Chemical Co., Milwaukee, WI) also was prepared in 50 mM MES, pH 7.0, at a concentration of 500 µg/mL. Equal volumes of the two reagents then were mixed to form a solution and immediately, 50 µL of the solution were applied to each silicon wafer, which had been activated following the procedure described in Example 1. The samples were allowed to react at room temperature for about 15 minutes and then were rinsed with distilled water and blown dry with a nitrogen stream as described in Example 1. The dry, highly reflective surface showed no visible residue.

Example 3

Reaction With rHBsAg

Test pieces with anti-HBsAg covalently attached and prepared as described in Example 2, were covered with 50 µL of a solution containing 200 µg/mL of recombinant Hepatitis B Surface Antigen subtype Ay (rHBsAg) (available from Abbott Laboratories, Abbott Park, IL in the commercially available kit Abbott IMx® AUSAB®) in 50 mM MES buffer pH 7.0, and allowed to react for approximately 20 minutes at room temperature. After reaction, the test pieces were washed with distilled water and dried with a stream of nitrogen gas as described in Example 1. Again, the dry, highly reflective surface appeared free of any visible residue.

Example 4

Ellipsometric Examination of Test Pieces

A test piece carried through the sequences described in Examples 1 and 2 was measured in a Rudolph Ellipsometer (model Auto EL, available from Rudolph Research, Flanders, NJ). Using a refractive index of 1.46, a film thickness of 37.7 Angstroms was obtained. Then, a test piece carried through the sequences described in Examples 1, 2, and 3 was measured in the Rudolph Ellipsometer. Again using a refractive index of 1.46, a thickness of 65.1 Angstroms was obtained. The thickness increase of 27.4 Angstroms was due to binding of the rHBsAg to the antibody coated surface of the test piece.

Example 5

Atomic Force Microscopy Examination of Test Pieces

An Atomic Force Microscope, built by TopoMetrix (Pasadena, CA) was used to scan the surfaces of the various test pieces produced according to Examples 1, 2 and 3 detailed hereinbelow in points a through d , as follows. A force of approximately 6 nN (nanoNewtons) was applied by the sensor which scanned across the surface at 500 nm/s (nanometers per second) in the X direction. Upon the completion of one pass, the sensor was advanced in a small direction in the Y direction, and another trace was made back in the X direction. The response of the sensor was monitored as it was rastered (i.e., systematically scanned) across the surface. The sensor response was utilized in a feedback loop to control the Z position of the sensor. In this fashion, a complete map was made of a function of the surface at various X, Y coordinates. Two hundred or four hundred line images were obtained.

a. A bare silicon test piece, which contained no antibody on it, was scanned. The surface appeared smooth and featureless.

b. A test piece prepared as described in Example 1 was scanned. FIG. 1A is an SPM image of the surface of the activated silicon test piece. Referring to FIG. 1A, the image shows a smooth, featureless surface. Three different lines (1, 2 and 3) were traced across the X, Y plane. Referring to FIG. 1B, which traces the apparent elevation of the probe above the surface across the X, Y plane at three different lines (1, 2 and 3) , the traces approximate a smooth catenary. The apparent increase in thickness at the edges of the trace are thought to be artifact.

c. A test piece prepared according to Example 2 was scanned. FIG.2A is an SPM image of the silicon test piece coated with an analyte specific substance (antibody). Referring to FIG. 2A, the image shows a smooth, essentially featureless surface. Three different lines (1, 2 and 3) were traced across the X, Y plane. FIG. 2B , which traces the apparent elevation of the probe across the X, Y plane at three different lines (1, 2 and 3) above the surface, the traces approximate a smooth catenary. The apparent increase in thickness at the edges of the traces are thought to be artifact.

d. A test piece prepared according to Example 3 was scanned and multiple fields were examined. FIG. 3A is an SPM image of a test piece coated with analyte-specific substance (antibody directed against Hepatitis B Surface Antigen [anti-HBsAg]) reacted with analyte (recombinant Hepatitis B Surface Antigen [rHBsAg]). Referring to FIG. 3A, the figure shows multiple antigen particles immobilized on the surface of the test piece. One major cluster of particles is seen approximately in the center with other particles in contact with another distributed around the image. Three different lines (1, 2 and 3) were traced across the X, Y plane. FIG. 3B gives three traces across the field. The upper trace (1) shows essentially constant elevation above the surface, while traces 2 and 3 traverse the large grouping of antigen particles. The artifact at the edges of the trace are still present. FIG. 4, which provides a different projection of a field, shows the image of multiple globular objects, crowded together, others presenting as isolated hemispheres when not in contact with other objects.

Thus, the method of the invention described herein can be used to assay for analytes which may be present in a test sample. It will be appreciated by those skilled in the art that many of the concepts of the present invention are equally applicable to other types of binding assays. The embodiments described and presented herein are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments disclosed, but it is intended to encompass all equivalents and subject matter within the spirit and scope of the invention as described and contemplated above, and as set forth in the following claims.

WHAT IS CLAIMED IS:

1. A method for determining the presence or absence of an analyte in a test sample, comprising:

a. contacting a test sample with a test piece to which an analyte specific substance has been attached for a time and under conditions sufficient to allow binding of the analyte to the test piece; and

b. scanning the test piece by scanning probe microscopy to determine the presence or absence of the analyte.

2. The method of claim 1, further comprising the step of quantitating the amount of analyte present by counting the number of analyte molecules in one or more fields of the test piece and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically.

3. The method of claim 1, wherein step (b) is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane such as to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates.

4. The method of claim 1 wherein said scanning is performed by a scanning tunnelling microscope.

5. The method of claim 1 wherein said scanning is performed by an atomic force microscope.

6. The method of claim 1 wherein said analyte specific substance is attached to the test piece by adsorption.

7. The method of claim 6 wherein said test piece comprises a solid phase of plastic or metal.

8. The method of claim 1 wherein said analyte specific substance is attached to the test piece by covalent binding.

9. The method of claim 8 wherein said test piece comprises a solid phase selected from the group consisting of derivatized plastic, metal, glass and silicon.

10. The method of claim 6 or 8 wherein said analyte specific substance is selected from the group consisting of an antibody, an antigen, an antigen analog, DNA, RNA, a lectin, a hapten, biotin, avidin, folate binding protein and intrinsic factor.

11. A method for determining the presence of an analyte suspected of being present in a test sample, comprising:

a. mixing the test sample with a known amount of its specific binding partner to form a mixture;

b. applying the mixture to the test piece to which the analyte or an analyte analog has been attached, for a time and under conditions sufficient for a reaction to occur; and

c. scanning the test piece by scanning probe microscopy to determine the presence and/or amount of the specific binding partner, wherein the presence and amount of the specific binding partner bound is inversely proportional to the amount of analyte present in the test sample.

12. The method of claim 11 further comprising the step of quantitating the amount of analyte present by counting the number of specific binding partner molecules in one or more fields of the test piece and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically.

13. The method of claim 11, wherein step (c) is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane such as to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates.

14. The method of claim 11 wherein said scanning is performed by a scanning tunnelling microscope.

15. The method of claim 11 wherein said scanning is performed by an atomic force microscope.

16. The method of claim 11 wherein said analyte or analyte analog is attached to the test piece by adsorption.

17. The method of claim 16 wherein said test piece comprises a solid phase of plastic or metal.

18. The method of claim 11 wherein said analyte or analyte analog is attached to the test piece by covalent binding.

19. The method of claim 18 wherein said test piece is selected from the group consisting of derivatized plastic, metal, glass and silicon.

20. The method of claim 16 or 18 wherein said analyte specific substance is selected from the group consisting of an antibody, an antigen, an antigen analog, DNA, RNA, a lectin, a hapten, biotin, avidin, folate binding protein and intrinsic factor.

21. A method for determining the presence and amount of an analyte in a test sample, comprising:

a. contacting a test sample with a test piece to which an analyte specific substance has been covalently attached for a time and under conditions sufficient to allow binding of the analyte to the test piece;

b. scanning the test piece by scanning probe microscopy to determine the presence or absence of the analyte; and

c. quantitating the amount of analyte by counting the number of analyte molecules in one more more fields and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically.

22. The method of claim 21, wherein step (b) is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane such as to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates.

23. The method of claim 21 wherein said scanning is performed by a scanning tunnelling microscope.

24. The method of claim 21 wherein said scanning is performed by an atomic force microscope.

25. A method of determining the presence and/or amount of an analyte which may be present in a test sample, comprising:

a. mixing the sample suspected of containing the analyte with a fixed amount of its specific binding partner to form a mixture;

b. applying the mixture to the test piece which is covalently bound with the analyte or an analyte analog for a time and under conditions sufficient for a reaction to occur; and

c. scanning the test piece by scanning probe microscopy to determine the presence and/or of the specific binding partner ;

d. quantitating the analyte by counting the number of molecules in a particular field and determining the concentration of analyte present in the test sample either by reference to previously measured standards or mathematically. wherein the presence and amount of the specific binding partner bound is inversely proportional to the amount of analyte present in the sample.

26. The method of claim 25, wherein step (c) is performed by monitoring the response of a microsensor as it is systematically scanned across a surface of the test piece in raster fashion to define an X, Y plane suchas to control the vertical displacement of the microsensor thereby making a map of a function of the surface of the test piece at various X, Y coordinates.

27. The method of claim 25 wherein said scanning is performed by a scanning tunnelling microscope.

28. The method of claim 25 wherein said scanning is performed by an atomic force microscope.

29. A test kit useful for determining the presence of an analyte suspected of being present in a test sample by scanning probe microscopy, comprising:

a test piece having bound thereto an analyte specific substance, an analyte, or an analyte analog.

30. The test kit of claim 29 wherein said analyte specific substance, analyte or analyte analog is covalently attached to said test piece.

31. The test kit of claim 29 wherein said test piece comprises a solid phase, said solid phase being selected from the group consisting of derivatized plastic, glass, silicon and metal.

32. The test kit of claim 31 wherein said test piece comprises a silicon wafer.

FIG. 1A

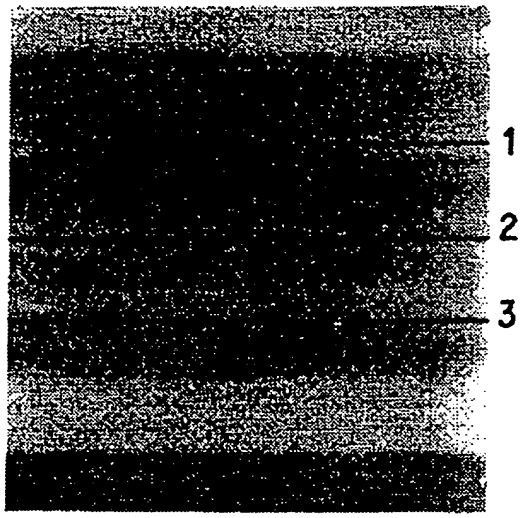


FIG. 1B

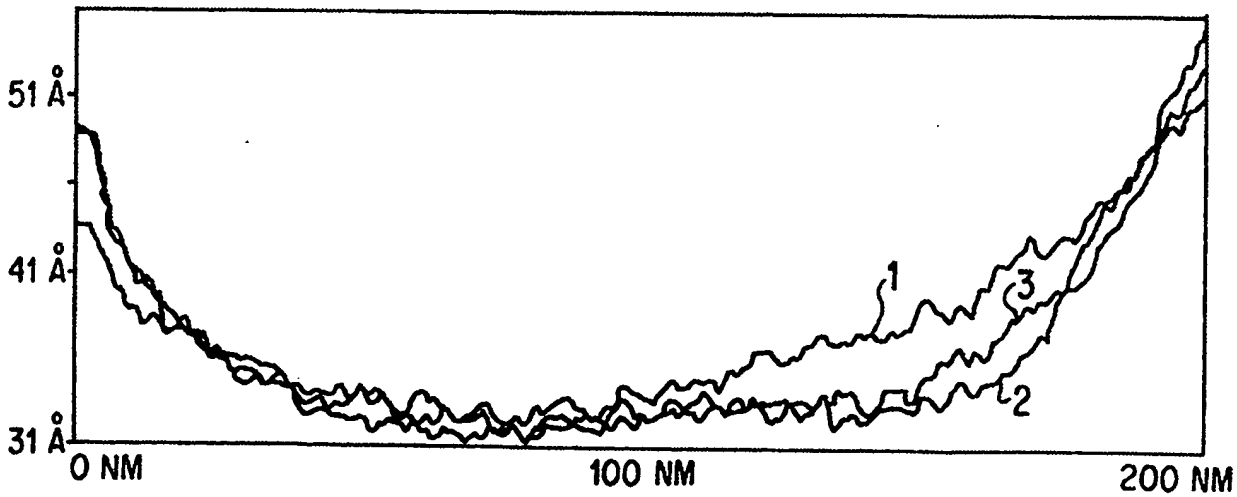


FIG. 2A

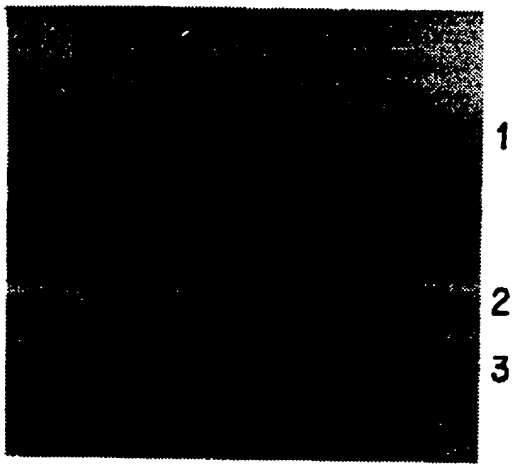


FIG. 2B

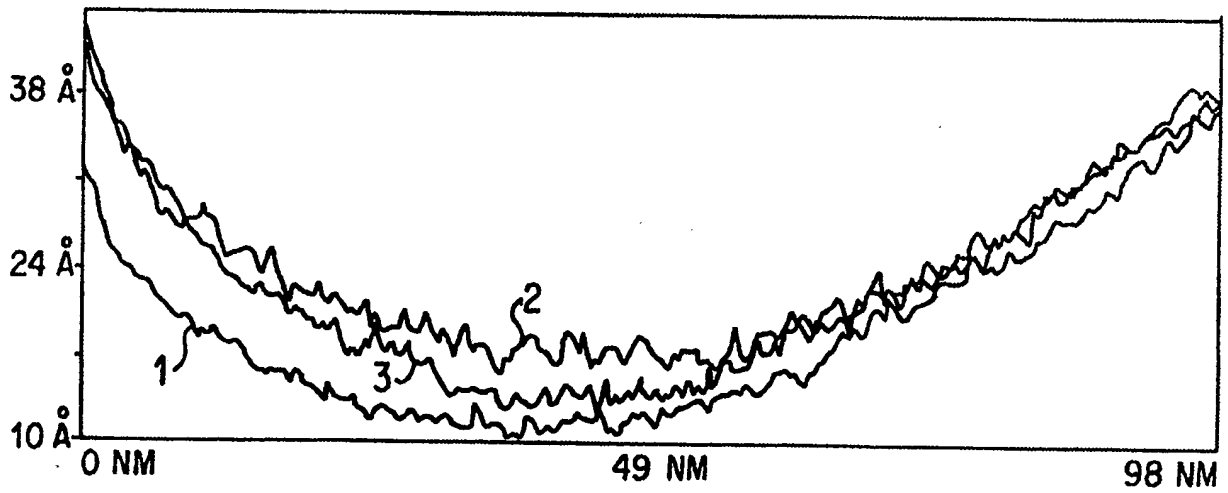


FIG. 3A

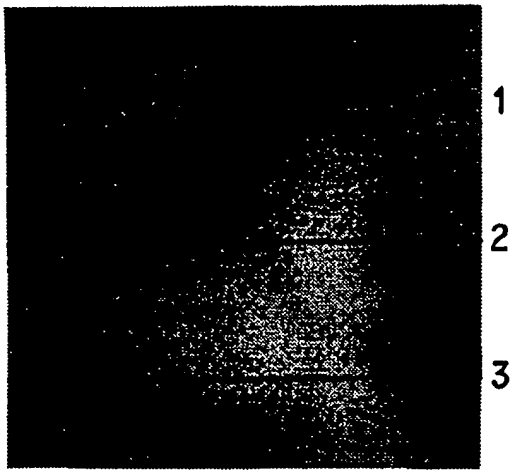
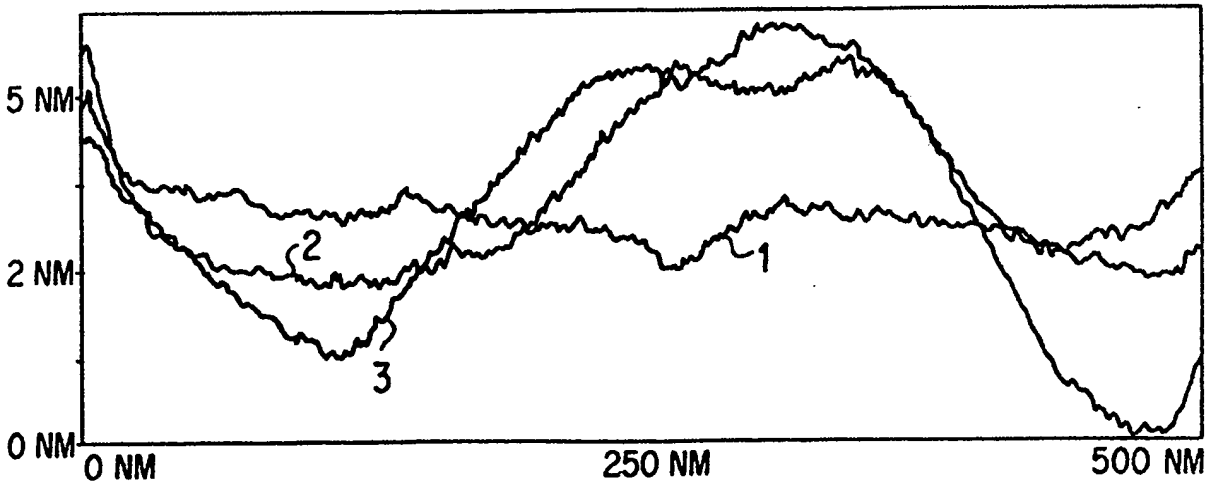


FIG. 3B



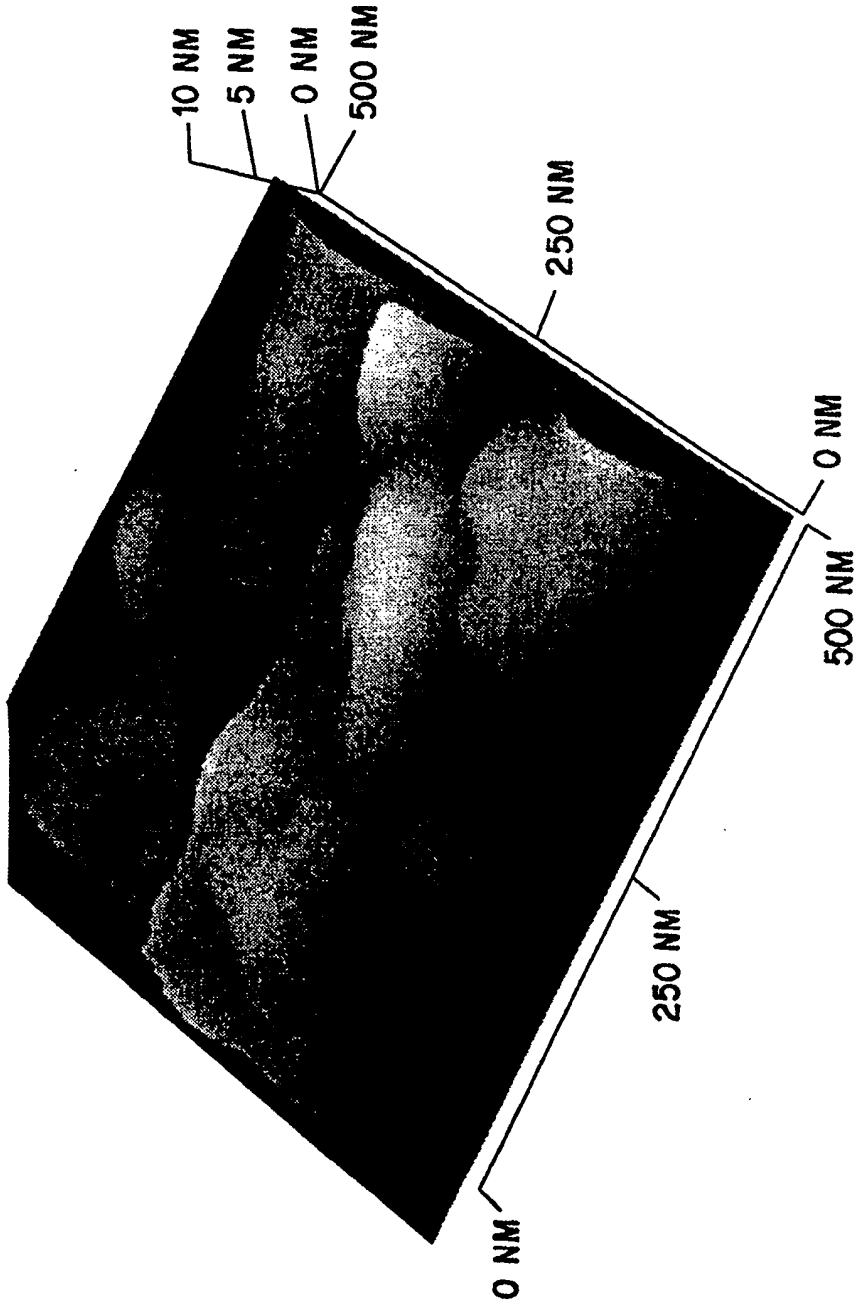


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01572

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): Please See Attached Sheet.
US CL : 435/6; 156/630, 633; 427/43.1; 430/5; 436/172

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6; 156/630, 633; 427/43.1; 430/5; 436/172

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
y,p	US, A, 5,047,633 (Finlan et al) 10, September 1991, see column 1, line 34; column 1, line 62; column 2, line 19; column 3, line 35	1-32
y	US, A, 4,833,093 (Malmqvist et al) 23 May 1989, see column 1, line 35; column 1, line 59.	29-32
y	Methods in Enzymology - Guide to Molecular Cloning Techniques, 1987, Jackson et al.. "In vivo footprinting of specific protein-DNA interactions" pages 735-755. Academic Press. See whole article.	11-28
y	Proc. Natl. Acad. Sci. USA, Volume 86, issued January 1989, Pihl et al, "Characterization of hydrogen-uptake activity in the hyperthermophile Pyrodictium brockii", pages 138-141, see page 138, col. 2.	1-32
y	Science, volume 243, issued Jan. 1989, Beebe Jr. et al, "Direct Observation of Native DNA Structures with the Scanning Tunnelling Microscope", pages 370-372. See whole article.	1-32

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
14 MAY 1992	26 MAY 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Deborah Fraud for Hyosuk Kim

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

a	Nucleic Acids Research, Volume 20, No. 3, issued February 1992, Henderson, Eric, "Imaging and nanodissection of individual supercoiled plasmids by atomic force microscopy", pages 445-447.	1-32
a	Science, Volume 244, issue June 1990, Lindsay et al, "Images of the DNA Double Helix in Water", pages 1063-1064.	1-32
a,p	Trends in Biotechnology, Vol. 9, issued June 1991, Walhlgren et al, "Protein adsorption to solid surfaces", pages 201-208.	1-32

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68; C03C 15/00; B05D 3/06; G03F 9/00; G01N 21/00, 21/66, 21/68, 21/75, 21/76

II. FIELDS SEARCHED
Other Documents Searched:

cas online, biosis, aps
search terms: atomic force microscopy, scanning probe microscopy, scanninig tunnelling microscopy, scanning force microscopy, immunoassay, hybridiz?, immuno?, antibod? or antigen?, dna or nucleic acid$, silicon, silic?, raster, protein or molecule or antibod?, stroupe